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Lipid Removal from Human Serum Samples

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The efficacy of lipid removal from human serum samples obtained by using Cleanascite HC, a commercially available product, was compared to that obtained by the standard chloroform method. Separate samples of 21 frozen, banked human serum samples used in the preparation of samples for proficiency testing were treated with either Cleanascite HC or chloroform. The lipid content was measured before and after treatment. The total percentages of lipid removed ranged from 61 to 70% with Cleanascite HC and from 60 to 62% with chloroform. The advantage of Cleanascite HC over chloroform is based on the simplicity of the procedure with Cleanascite HC without the environmental concerns inherent in the use of chloroform. In 15 serum samples known to contain antibodies to treponemal and nontreponemal syphilis antigens, Cleanascite HC bound some immunoglobulin, but with only minimal loss of reactivity in the serologic tests for syphilis. Cleanascite HC is therefore an acceptable alternative to chloroform for lipid reduction in human serum samples.

Human sera used in the preparation of syphilis serology reference controls or samples for proficiency testing generally have high lipid contents. The presence of excess lipids in these sera is objectionable because of the unaesthetic appearance, difficulty in rehydration after lyophilization, and possible interference in the nontreponemal tests for syphilis. Traditionally, chloroform has been the preferred method for delipidization of human or animal sera used in the manufacturing of diagnostic or control reagents. Although chloroform effectively removes lipids, its use is not advisable because of environmental concerns. Chloroform is classified as a carcinogen and requires both monitoring of personnel exposure time and hazardous waste disposal. Chloroform use is also inconvenient due to the amount of labor and time required for emulsification and separation. Cleanascite HC consists of moderately hydrophobic silica which has been wetted or "activated" so that it will disperse in aqueous media. This permits productive interaction with lipophilic biomolecules, presumably by the release of water from the surface. The surface structure has also been modified by a proprietary process in order to minimize nonspecific interactions with proteins (4). Cleanascite HC is supplied as a finely distributed, solid-phase suspension (33% centrifuged volume/total volume) in saline. When human sera are treated with Cleanascite HC, lipids are removed at a ratio similar to or better than that obtained with chloroform, with only a minimal loss of reactivity of the antisera due to immunoglobulin G (IgG) or IgM binding.

The purpose of this study was to evaluate Cleanascite HC treatment as an alternative to chloroform treatment for the removal of lipids from frozen, banked sera. Fresh serum samples from syphilis patients were not included in this initial study. As part of the evaluation, we determined the decreases in the amounts of total lipid and protein and any effect on the reduction of reactivity in the treponemal and nontreponemal tests for syphilis.

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MATERIALS AND METHODS

Serum sample treatment. Twenty-one separate human serum samples which had been stored in bulk at −20°C for 1 to 18 years were treated with either Cleanascite HC (Affinity Technology, Inc., Fairfield, N.J.) or chloroform. Fifteen of the serum samples contained both treponemal and nontreponemal antibodies. The remaining six serum samples were nonreactive in all tests for syphilis. We added 1 ml of Cleanascite HC to each of 21 glass test tubes (12 by 75 mm) and centrifuged them at 1,000 × *g* for 20 min. The supernatant was decanted, and 2 ml of the serum to be treated was added to the Cleanascite HC pellet. These tubes were vortexed to suspend the pellet and were then incubated at 2 to 8°C overnight with constant gentle agitation at approximately 27 rpm on a tabletop rocker platform. Following incubation, the samples were centrifuged at 1,000 × *g* for 45 min. The treated sera were decanted into another set of correspondingly labeled glass test tubes (12 by 75 mm). The sera were then filtered through 0.45-μm-pore-size filter membranes (Gelman Sciences, Ann Arbor, Mich.) to remove any broken polymer particles that might be in the suspension.

For chloroform extraction, 1 ml of each serum sample was added to a second set of glass test tubes. One milliliter of chloroform was then added to each tube and the tube was vigorously vortexed until a thick emulsion was obtained. The tubes were then centrifuged at 1,000 × *g* for 30 min. The supernatant was removed from the lipid-chloroform layer by decanting it into correspondingly labeled microcentrifuge tubes (39 by 10 mm; Sarsted, Newton, N.C.), which were then centrifuged at 10,000 × *g* for 45 min. The supernatant was carefully decanted into corresponding glass test tubes (12 by 75 mm) (10).

Sample testing. Total lipid determination was made for all the serum samples, including the pretreatment sample, by two different methods. Total cholesterol was determined enzymatically by a modification of the method of Allain et al. (1). Triglycerides were measured by using a quantitative enzymatic means of determination of the glycerol level, as modified by McGowan et al. (8). Total serum lipid levels (expressed as milligrams per deciliter) were calculated by using the formula TL = 2.27 TC + TG + 0.623, where TL is the total lipid level, TC is the total cholesterol level, and TG is the total triglyceride level (9).

The protein concentration was determined by the method of Bradford (2) (Bio-Rad Laboratories, Richmond, Calif.). Total IgG and IgM for each of the 15 syphilis-reactive serum samples was determined by radial immunodiffusion (3, 6, 7) (The Binding Site, Birmingham, United Kingdom).

Serologic testing for syphilis was done with each set of the 21 samples treated with Cleanascite HC and chloroform. The tests performed were (i) the rapid plasma reagin circle card test (RPR), (ii) the Venereal Disease Research Laboratory test (VDRL), (iii) the unheated serum reagin test (USR), and (iv) the toluidine red unheated serum test (TRUST). All tests were performed by standard methods (5). The treponemal tests done were (i) the fluorescent treponemal antibody-absorption double staining test (FTA-ABS DS) (5), (ii) the microhemagglutination assay for *Treponema pallidum* (MHA-TP) (5), and (iii) the Captia Syphilis-G and Syphilis-M enzyme immunoassay (EIA; Sanofi Diagnostics Pasteur, Chaska, MN) for IgG and IgM antitreponemal antibodies, respectively.

RESULTS

Cleanascite HC removed, on average, a higher percentage of lipid than the chloroform method did and was more efficient at lipid removal (70.48 versus 62.26%) when it was used with sera

TABLE 1. Human sera treated with Cleanascite HC and chloroform

Reactivity by STS	Avg age of serum (yr)	No. of serum samples tested	% Avg lipids removed		% Avg protein content removed		% Avg total IgG removed		% Avg total IgM removed	
			Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform
Reactive	1.5	8	70.48	62.26	19.21	9.42	20.56	3.38	21.57	4.80
Minimally reactive	18	7	67.49	62.19	15.30	5.18	22.27	5.79	13.43	6.20
Nonreactive	6	6	61.62	60.24	12.93	3.07	ND[a]	ND	ND	ND
Total		21	66.95	61.66	16.11	6.19	21.36	4.50	17.77	5.45

[a] ND, not determined.

that had been frozen for 2 years or less. For sera that had been stored for longer periods (average, 18 years), the efficiencies of lipid removal were 67.49% by the Cleanascite HC method and 62.19% by the chloroform method (Table 1).

There was greater protein loss with samples treated with Cleanascite HC (12.93 to 19.21%) compared to the loss with samples treated with chloroform (3.07 to 9.42%), possibly due to the removal of lipoprotein by Cleanascite HC (Table 1).

The 15 samples that were reactive in the serologic test for syphilis (STS) were tested for total IgG and IgM concentrations. Samples treated with Cleanascite HC demonstrated a reduction in IgG content of 20.56 to 22.27%, while samples treated with chloroform showed a reduction in IgG content of 3.38 to 5.79%. Serum samples treated with Cleanascite HC showed a quantitative IgM reduction of 13.43 to 21.57%, whereas samples treated with chloroform showed a quantitative IgM reduction of 4.80 to 6.20% (Table 1).

To determine the effect of Cleanascite HC in reducing specific antibody content, we tested 15 of the reactive serum samples using the treponemal and nontreponemal tests for syphilis. In the nontreponemal tests (RPR, TRUST, VDRL, and USR), both the treated and untreated sera were tested in parallel and the endpoint titers were determined (Table 2). By RPR, an endpoint titer of 1 dilution less was found for four samples treated with Cleanascite HC and three samples treated with chloroform. By USR, five serum samples treated with Cleanascite HC and three serum samples treated with chloroform had 1 doubling dilution reductions in titer. By TRUST, seven samples treated with Cleanascite HC and two samples treated with chloroform showed 1 doubling dilution reductions in antibody titers. By VDRL, five samples treated with chloroform and four samples treated with Cleanascite HC demonstrated twofold antibody titer losses. No serum sample became nonreactive by any test when it was subjected to either treatment.

The same 15 reactive and minimally reactive serum samples were tested qualitatively by FTA-ABS, MHA-TP, and the EIAs for IgG and IgM antibodies (Table 3). By FTA-ABS and MHA-TP, no change in reactivity was found for any of the samples treated with chloroform or Cleanascite HC, although five samples treated with chloroform showed a slight decrease in fluorescence intensity by FTA-ABS. By the EIAs, the antibody index is used to determine whether the sample is reactive, equivocal, or nonreactive. By the EIA for IgG antibody against *T. pallidum* antigen, the result was changed from reactive to equivocal for one sample treated with Cleanascite HC but for none of the samples treated with chloroform. By the EIA for IgM antibody against *T. pallidum* antigen, no result was changed by Cleanascite HC treatment, but the result for one sample treated with chloroform changed from reactive to equivocal and that for another sample changed from equivocal to nonreactive.

DISCUSSION

For all 21 samples tested, lipid removal was more effective with Cleanascite HC (61.62 to 70.48%) than with chloroform (60.24 to 62.26%). The length of time that the serum is stored may affect treatment with Cleanascite HC, since lipid removal was more efficient (70.48%) for sera stored for 2 years or less. Of the 21 samples tested, 8 had been stored at −20°C for 1 to 2 years, while the rest had been stored for 6 to 18 years. The

TABLE 2. Effect of treatment on reactive and nonreactive sera in the nontreponemal tests for syphilis

Reactivity by STS and titer difference	No. of serum samples tested	No. of serum samples							
		RPR		TRUST		VDRL		USR	
		Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform
Reactive	8								
Endpoint titer equal to that before treatment		7	7	5	8	6	6	7	7
Endpoint titer 1 dilution less than that before treatment		1	1	3	0	2	2	1	1
Minimally reactive	7								
Endpoint titer equal to that before treatment		4	5	3	5	5	4	3	5
Endpoint titer 1 dilution less than that before treatment		3	2	4	2	2	3	4	2
Nonreactive	6	Neg[a]	Neg	Neg	Neg	Neg	Neg	Neg	Neg

[a] Neg, negative result.

TABLE 3. Effect of treatment on reactive and nonreactive sera in the treponemal tests for syphilis

Reactivity by STS and reaction difference	No. of serum samples tested	No. of serum samples							
		FTA-ABS		MHA-TP		EIA for IgG		EIA for IgM	
		Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform	Cleanascite HC	Chloroform
Reactive	8								
Reaction equal to that before treatment		8	8	8	8	7	8	8	7
Reaction less than that before treatment		0	0	0	0	1[a]	0	0	1[a]
Minimally reactive	7								
Reaction equal to that before treatment		7	7	7	7	7	7	7[b]	6
Reaction less than that before treatment		0	0	0	0	0	0	0	1[c]
Nonreactive	6	Neg[d]	Neg	Neg	Neg	Neg	Neg	Neg	Neg

[a] Reaction changed from reactive to equivocal.
[b] Four of seven samples were nonreactive before and after treatment.
[c] Reaction changed from equivocal to nonreactive.
[d] Neg, negative result.

effect of storing treated samples at −20°C is not known but is being evaluated.

The total protein content of the samples tested indicated some loss of protein after treatment with Cleanascite HC, which may be due to the removal of lipoproteins. The loss of total immunoglobulin is meaningful if the specific antibody content of the samples is reduced to a point which affects the desired antibody titer. The antigens for all the nontreponemal tests (VDRL, RPR, USR, and TRUST) are based on the VDRL antigen, which is an alcoholic solution that contains cardiolipin, lecithin, and cholesterol. The antigen flocculates when it is combined with nontreponemal antibodies in serum. Excessive lipids in the serum can interfere with the interpretation of nontreponemal test results, especially in sera stored for long periods, which have the tendency to develop lipoidal clumps. We obtained a more accurate assessment of the effect of lipid removal on antibody reduction in these tests by comparing endpoint titers before and after treatment. Some sera had a 1-doubling-dilution loss of antibody titer after treatment with either Cleanascite HC or chloroform, but none of the STS-reactive samples became nonreactive after treatment and none of the STS-nonreactive samples became reactive after treatment.

By the EIAs, the antibody index, which is used to determine whether a serum sample is reactive, equivocal, or nonreactive, decreased after treatment with Cleanascite HC, demonstrating some loss of specific immunoglobulin content. This was more evident in the test for IgG, with a change in results from reactive to equivocal for one serum sample. By contrast, in the test for IgM chloroform treatment appeared to have more of an effect, with two of the serum samples changing from equivocal to nonreactive after being treated. Since the EIAs are considered qualitative rather than quantitative, decreases in antibody index are not considered important when they do not affect qualitative results.

Some decreases in the intensities of reactivities by MHA-TP and FTA-ABS occurred for the serum samples treated with chloroform but not for those treated with Cleanascite HC. This would suggest that Cleanascite HC would be preferred over chloroform for the treatment of sera with a 1+ to 2+ agglutination or fluorescence since chloroform treatment might remove the reactivity.

Because filtration to reduce lipid agglomerates in serum is inefficient and organic solvents such as chloroform are considered toxic and create hazardous waste-disposal problems, we sought an alternative method for lipid removal that was both safe and effective. Our studies indicate that Cleanascite HC meets these criteria for removal of excess lipids from reference control sera or samples to be used for proficiency testing. It is important that serum which is not cloudy be used to prepare these samples, since cloudiness causes some end users to think that the samples are contaminated. No serum became completely nonreactive as a result of the treatment, and any decreases in nontreponemal antibody titer were usually within a 1 doubling dilution, which we do not consider a significant decrease since this is within the allowable margin of error for the tests.

We conclude that the use of Cleanascite HC for reducing excessive amounts of lipids in frozen, banked sera is a good alternative to chloroform treatment when preparing serum sample panels for use as controls, as reference standards, or for proficiency testing.

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